A DNA MOLECULE ENCODING A VARIANT PARAOXONASE AND USES THEREOF

FIELD OF THE INVENTION

The present invention relates to a DNA molecule encoding a variant human paraoxonase (EC 3.1.1.2), and to said variant paraoxonase protein. The present invention also relates to a method for detecting or predicting the risk of, or predisposition to, cancer, coronary and cerebrovascular diseases, type 2 diabetes, hypertension, dementia, arthrosis, cataract and sensitivity to organophosphorus compounds in a subject, as well as to a kit or assay for carrying out the said method. This invention also relates to targeting paraoxonase enhancing treatments and to transgenic animals comprising a human DNA molecule encoding said variant paraoxonase and to a method of mutation search.

BACKGROUND OF THE INVENTION

The publications and other material used herein to illuminate the background of the invention are incorporated by reference.

Oxidative stress and free radicals have been implicated in the etiology of a number of diseases, including cancers, coronary heart disease, cerebrovascular disease, type 2 diabetes, hypertension, dementia and cataract. The human body has a number of endogenous free radicals scavenging systems which have genetic variability. The human serum paraoxonase (PON) is an enzyme carried in the high-density lipoprotein that contributes to the detoxification of organophosphorus compounds but also of toxic products of lipid peroxidation.[1-9] The paraoxonase hydrolyzes the toxic metabolites of several organophosphorus (OP) insecticides, pesticides and nerve agents.

The *PON1* gene is polymorphic in human populations and different individuals also express widely different levels and activities of the paraoxonase enzyme, which is the protein product coded by the gene.[3,5-7]

Several polymorphisms are currently known in human *PON1*. The Gln191Arg polymorphism was the first mutation of *PON1* reported.[3,6] The second one is the Met54Leu.[3] Both these polymorphisms have been shown to affect serum PON activity.[6,10,11]

Transgenic animals and with lowered paraoxonase activity can be used e.g. to test the effects of organophosphorus compounds, such as insecticides, pesticides and war agents, drugs that affect paraoxonase activity, other antioxidative compounds and drugs, and liver enzyme activity inducing agents.

A lot of methodological work has been done to locate disease-causing genes or candidate genes. However, there are no previous methodological studies concerning the methods of how to promote the search for mutations in a given or known candidate gene. To facilitate the finding of mutant DNA sequences, we developed a new method of phenotype-targeted gene sequencing.

SUMMARY OF THE INVENTION

One object of this invention is to provide a DNA sequence of a variant human *PON1* gene and the amino acid sequence of the corresponding variant paraoxonase protein. Another object of the invention is to provide a method for screening a subject to assess if such subject is at risk of cancer, coronary or cerebrovascular disease, hypertension, type 2 diabetes, dementia, joint arthrosis or eye cataract, or at risk of being sensitive to organophosphate toxicity. The invention is also directed to a kit or an assay for said method, as well as to a probe for use in said method or kit. A further object of the invention is to provide a method for targeting a paraoxonase enhancing treatment for example for the above mentioned diseases and for organophospate poisoning, and/or for assessing the effectiveness of paraoxonase modifying treatments. A fourth object of the invention is to provide a transgenic animal with a gene encoding a variant paraoxonase. A fifth object of the invention is to provide a method for rapid search of gene mutations. These and further objects will be evident from the following description and claims.

According to one aspect, the invention concerns a DNA sequence comprising a nucleotide sequence encoding a variant paraoxonase protein with the Ile102Val mutation. The said mutation can, in the alternative, be named also Ile101 Val, if the start codon atg (Met) is not included in the count. In the following description and claims, reference is made to the Ile102Val mutation, but said reference means within the scope of the invention in the alternative the Ile101Val mutation in case the alternative way of counting is used. The invention also concerns a variant paraoxonase protein with the Ile102Val mutation.

According to further aspect, the invention concerns a method for screening a subject to determine if said subject is a carrier of a variant gene encoding a variant paraoxonase, by determining the allelic pattern for the codon 102 of the human *PON1* gene, i.e. to determine if the said subject is a carrier of the Ile102Val mutation.

Specifically such a method comprises the steps of

a) providing a biological sample of the subject to be screened, and

b) providing an assay for detecting in the biological sample the presence of the Ile102Val or Val102Val genotype of the human *PON1* gene.

The assay result can be used for assessing the subject's risk to develop a low paraoxonase expression related disease such as cancer, coronary or cerebrovascular disease, type 2 diabetes, hypertension, dementia, arthrosis or cataract or sensitivity to organophosphorus compounds, and/or for assessing the effectiveness of paraoxonase-inducing therapy in a subject, whereby identification of a Ile102Val mutation being indicative of said risk being increased or effectiveness being modulated.

The present invention is thus directed to a method for detecting a risk of cancer, coronary or cerebrovascular disease, type 2 diabetes, hypertension, dementia, arthrosis or cataract in a subject, comprising isolating genomic DNA from said subject, determining the allelic pattern in the exon number 4 in the codon number 102 of the paraoxonase encoding *PON1* gene in the genomic DNA, and identification of Ile102Val mutation indicating said risk being increased.

The present invention is also directed to a method for assessing the effectiveness of paraoxonase inducing therapy of a subject, comprising isolating genomic DNA from said subject, determining the allelic pattern in the exon number 4 in the codon number 102 of the paraoxonase encoding *PON1* gene in the genomic DNA, and identification of Ile102Val mutation indicating said effectiveness being modulated, e.g. reduced.

The invention is also directed to a method for determining the presence or absence in a biological sample of a DNA sequence comprising a nucleotide sequence encoding a variant paraoxonase protein, the method comprising isolating genomic DNA from said subject, determining the allelic pattern in the exon number 4 in the codon number 102 of the paraoxonase encoding *PON1* gene in the genomic DNA, and identification of Ile102Val mutation indicating the presence of said DNA sequence.

The techniques for carrying out such a method and presented here are intended to be non-limiting examples. One skilled in the art will readily appreciate that other methods for detection of the variant DNA sequence can be used, developed or modified.

One detection method is minisequencing which is based on a minisequencing reaction, in which an oligonucleotide that ends one nucleotide upstream the variant nucleotide, is enzymatically elongated by one nucleotide that is complementary to either the variant or the wild type nucleotide in the target sequence, and this added labelled nucleotide is detected. Such label can be, for example, radioactive or fluorescent label.

Another detection method is based on appearance or disappearance of an enzymatic cleavage site by the variant nucleotide. This kind of detection can be performed by first amplificating the target nucleotide sequence by a polymerase chain reaction with primers that flank the variant nucleotide, and then digesting the reaction product with a restriction endonuclease that recognises only the variant or only the wild- type sequence, producing DNA fragments of different length for each. These fragments may be recognised, for example, by gel electroforesis with DNA staining.

Yet another detection method is the oligonucleotide ligation assay, in which two allele specific oligonucleotide probes and one common oligonucleotide probe are used to distinguish between the variant and wild-type nucleotide. In this method, the target sequence is hybridised with the three oligonucleotide probes, and the probe pair that is complementary to the target sequence is joined enzymatically at the site of the variant nucleotide. The detection of the two alleles is based on differing labels, for example fluorescent labels of different colour, of the two allele specific oligonucleotide probes.

Furhermore, a detection method is the single stranded conformational analysis, in which the different alleles of a target sequence are identified on the basis of a difference in the electrophoretic mobility of the two alleles. In this method, the variant and wild-type target sequences that are in single stranded form, migrate with different speed through an electrophoresis matrix. Preferably, the target sequence is first amplified with a polymerase chain reaction, and the product is labelled for detection by radioactive or fluorescent label.

Yet furthermore, a detection method is sequencing, in which each nucleotide of the target sequence is identified. The variant allele is identified by the variant nucleotide.

Another detection method is allele specific hybridisation, in which an oligonucleotide probe is hybridised with the target sequence, and in which the probe is complementary only to the variant or wild-type allele. Preferably, two allele specific probes are used simultaneously to identify both alleles. Detection of a successful hybridisation and the determination of a genotype is based on detection of the probe-target duplex, on a basis of enzymatic colour reaction, or based on a label on the probe or on the target, for example a radioactive or a fluorescent label.

The present invention is also directed to a kit or assay for detecting a risk of cancer, coronary or cerebrovascular disease, type 2 diabetes, hypertension or dementia and sensitivity to organophosphorus compounds, and/or for assessing the need for or effectiveness of paraoxonase inducing therapy in a subject, comprising means for determining the allelic pattern in the exon number 4 in the codon 102 of the

paraoxonase encoding *PON1* gene in a genomic DNA sample. The assay may be a part of a DNA macroarray or microarray or a DNA chip or a DNA slide, which is intended for the detection of multiple gene mutations.

According to a further aspect, the present invention concerns a transgenic animal which carries a human DNA sequence comprising a nucleotide sequence encoding a variant human paraoxonase protein.

According to a further aspect, the present invention concerns the method of phenotype-targeted gene sequencing.

DETAILED DESCRIPTION OF THE INVENTION

In order to find new previously unknown functional mutations in the human *PON1* gene, phenotype-targeted hierarchial sequencing was used. The serum paraoxonase activity was determined for over 1000 serum samples. DNA samples of 10 persons with the lowest PON activity were first chosen for sequencing and they were sequenced through in all 9 exons with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). A new previously unknown human *PON1* mutation was found in codon number 102 in exon number 4, called PON Ile102Val, causing the change ATC to GTC; Ile to Val. After the new mutation was found, DNA samples of 100 men with low paraoxonase activities were sequenced, and the mutation was present in 9.0 % of the subjects. Finally 1,595 DNA samples available in the KIHD (Kuopio Ischaemic Heart Disease Risk Factor Study) cohort were genotyped and the new mutation was found for 61 persons; 3.8% of the random population sample of men.

A polymerase chain reaction was carried out as follows: the genomic DNA was amplified in eight parts specific for the *PON*1-gene and for its exons 1 to 9. Eight different amplifications were made, with eight different PCR primer pairs (SEQ ID NO:5-20); one pair for each exon except for the exons 2 and 3 which were amplified together. All 9 exons were sequenced.

The kit or assay for use in the method according to the invention preferably contains the various components needed for carrying out the method packaged in separate

containers and/or vials and including instructions for carrying out the method. Thus, for example, some or all of the various reagents and other ingredients needed for carrying out the determination, such as buffers, primers, enzymes, control samples or standards etc can be packaged separately but provided for use in the same box. Instructions for carrying out the method can be included inside the box, as a separate insert, or as a label on the box and/or on the separate vials.

EXPERIMENTAL SECTION

Polymerase chain reaction

The method according to the invention for determining the allelic pattern of the codon in question is preferably carried out as a polymerase chain reaction, in accordance with known techniques.[3] The PCR primer pair for human paraoxonase (PON 1) exon number 4 was as follow: 5′-CTCCTCCATGGTTATAAGGG-3′ (SEQ ID NO:9) and 5′-CCCAGAGTAAGAACATTATTC-3′(SEQ ID NO: 10) (product size 315 bp). The primers were designed by Marja Marchesani and they were delivered by the AIV Institute, sequencing services (Kuopio, Finland). PCR amplification was conducted in a 25 µl volume containing 150 ng genomic DNA (extracted from peripheral blood), 10 x PCR buffer, dNTP (10 mM of each), 20 pmol/µl of each primer, DNA-polymerase (2U/µl) (DyNAzymeTM DNA polymerase kit, Finnzymes, Espoo, Finland). Samples were amplified with a Biometra UNO programmable thermoblock (Biometra, Göttingen, Germany) with PCR programme conditions as follows: 95° C for 3 minutes, Repeat following for 30 cycles: 95° C for 30 seconds, 58° C for 45 seconds, 72° C for 45 seconds, 72° C for 5 minutes, 4° C hold. Amplified PCR-products were purified using the QIAquik PCR purification kit (QIAGEN, Valencia, CA).

Sequencing

Sequencing was made using a ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The ABI PRISM® 3100 Genetic Analyzer is a fluorescence-based DNA analysis system of capillary electrophoresis with 16 capillaries operating in parallel, fully automated from sample loading to data analysis.

The sequencing reactions were made by using the DNA Sequencing Kit; Big Dye TM Terminator cycle sequencing v.2.0 ready reactions with ampliTaq® DNA polymerase (Fs ABI PRISM®, PE Biosystems, Foster City, CA). The sequencing primers were the same as the PCR primers: 5′-CTCCTCCATGGTTATAAGGG-3′ (SEQ ID NO:9) or 5′-CCCAGAGTAAGAACATTATTC-3′ (SEQ ID NO: 10). Cycle sequencing was made in the GeneAmp PCR System 9600 (PE Biosystems) with the programme as follows: Repeat the following for 25 cycles; rapid thermal ramp to 96° C, 96° C for 10 seconds, rapid thermal ramp to 50° C, 50° C for 5 seconds, rapid thermal ramp to 60° C, 60° C for 4 minutes (to perform cycle sequencing under standard conditions, ABI PRISM® 3100 Genetic Analyzer Sequencing Chemistry Guide, Applied Biosystems).

Dye Terminator Removal and sequencing reaction clean-up was made using multiscreen 96-well filtration plates (Multiscreen® -HV clear plates, Millipore, Bedford, MA). After purification the samples were denaturated at 94° C for 1 min and the sequencing was done using the ABI PRISM® 3100 Genetic Analyzer using MicroAmp optical 96-well reaction plates (Applied Biosystems).

Genotyping

Specifically genotyping was done by extracting DNA from EDTA blood with a salting-out method after lysing red cells with10mM NaCl/10 mM EDTA. The 315 bp exon 4 PCR-product of the *PON1* gene was digested with Sau 3 AI restriction endonuclease (New England BioLabs, Beverly, MA), mixed with 6x loading dye solution and run in 2.0 % agarose gel electroforesis. Identification of normal and mutant forms was based on different electrophoretic migration rates of the restriction fragments, resulting in distinct bands (normal form (Ile102Ile);196 bp,100 bp,19 bp, heterozygote form (Ile102Val); 215 bp,196 bp,100 bp,19 bp and homozygote form (Val102Val); 215 bp,100 bp).

Determination of serum PON activity

Serum paraoxonase activity was measured based on its capacity to hydrolyse paraoxon. 100 µl of diluted serum (25-fold dilution in TRIS-HCl buffer, pH 8.0) was

mixed with 100 µl of paraoxon (Paraoxon, Dr. Ehrensdorfer GmbH, Augsburg, Germany) (0.1g in 66.1 ml of TRIS-HCl buffer, pH 8.0). Formation of p-nitrophenol was monitored photometrically at 405 nm (at 30C), as previously described .[12]

Testing for the risk of cancer, coronary or cerebrovascular disease, type 2 diabetes or hypertension

The study subjects were from the "Kuopio Ischaemic Heart Disease Risk Factor Study" (KIHD), a prospective population study to investigate risk factors for cardiovascular diseases, type 2 diabetes, hypertension, dementia and cancers.[13-17,19,20] The KIHD study protocol was approved by the Research Ethics Committee of the University of Kuopio, Finland. The study sample comprised men from Eastern Finland aged 42, 48, 54 or 60 years. A total of 2,682 men were examined during 1984-89. All participants gave a written informed consent. A DNA sample was available for 1595 men.

All cancer cases in the health care have been reported to a national cancer registry in Finland since 1953.[18] Our study cohort was record-linked to this cancer registry data by using the unique personal identification code (social security number) that all Finns have. Deaths in the cohort were obtained by record linkage to the national death certificate registry and hospitalizations by record linkage to the national hospital discharge registry. The history of hypertension and diabetes was assessed at baseline and at a 4-year follow-up by self-administered questionnaire, checked by an interviewer. Both at baseline and at the 4-year follow-up examination, blood pressure and fasting blood glucose were measured using identical methods both at baseline and at the 4-year follow-up.[16,20]

The first occurrence of cancer after the KIHD baseline examination was registered in the cancer registry during 1984-97 for 60 cohort members. The primary site was prostate for 15 cancers. There were 1246 men with no prior CHD or cerebrovascular disease. Of these, 342 were smokers and 904 non-smokers. Of the smokers, 21 died of a cardiovascular cause by the end of 1998. Of the 515 men examined at baseline during 1984-86, 36 developed an arthrosis (ICD-10 M15-M19) by the end of 1998. Of

the 1107 non-smoking men, 23 developed a cataract (ICD-10 H26-H29) by the end of 1998.

The association of the *PON1* Ile102Val genotype with the risk of hypertension and diabetes was studied among 1038 men who were re-examined 4 years after the baseline examination, see references 15,19 for details of the re-examination. For the analysis of the incidence of hypertension, hypertensive (history of hypertension, antihypertensive medication or systolic BP 160 mmHg or more or diastolic BP 95 mmHg or more) and obese (body mass index 29 kg/m^2 or more) men and those with a history of cancer were excluded, leaving 488 men for the analysis. For the analysis of the incidence of type 2 diabetes, men with a history of cancer or prevalent diabetes at baseline (fasting blood glucose 6.7 mmol/l or more or treatment for diabetes) were excluded, after which exclusion there were 967 men for the analysis.

Lipoproteins were separated from fresh serum samples using ultracentrifugation and precipitation.[13,14] Cholesterol and triglyceride concentrations were measured enzymatically, plasma ascorbate and lipid-standardized plasma vitamin E concentration by HPLC methods[16,20] serum ferritin and apolipoproteins with a RIA[12]. The maximal oxygen uptake, a measure of cardiorespiratory capacity, was measured directly during a symptom limited exercise test.[15] Information regarding medical history and medications was obtained by interview. Smoking was recorded using a self-administered questionnaire and the dietary intake of nutrients was estimated by four-day food recording.[17]

Risk-factor adjusted relative risks of cancer, prostate cancer and cardiovascular death were estimated by multivariate Cox proportional hazards modelling and those of incident hypertension and incident diabetes by multivariate logistic regression modelling. Covariates were selected by forward step-up modelling, using P-value of 0.10 as entry criterium. Missing values in covariates were replaced by grand means. Tests of statistical significance were one-sided. The statistical analyses were performed with SPSS version 10.0 for Windows.

Of all members of the study cohort, 61 (3.8 %) were Val allele carriers of the *PON1* gene Ile102Val polymorphism. To ascertain the penetrance of the *PON1* 102 mutation, serum PON activity was measured at the 11-year re-examination for 783 cohort members as described above. The mean activity was 168.7 U/l in the wild Ile-Ile homozygotes vs. 70.7 U/l in 102Val carriers ($p<0.001$). In a 2-way analysis of variance (n=782), the Ile102Val polymorphism ($p<0.001$) was a stronger predictor of paraoxonase activity than the Leu54Met polymorphism ($p=0.016$).

In a multivariate Cox model adjusting for the strongest other risk factors in this cohort: maximal oxygen uptake, dietary vitamin C intake, smoking status (current smoker vs. non-smoker), body mass index, serum lipoprotein (a), dietary iron intake and apolipoprotein B, the relative risk of any cancer in the 102Val carriers was 2.4 (90% CI 1.0 to 5.5, $p=0.052$), compared with 102Ile homozygotes ($p<0.001$ for the model, Table 1). This association was stronger in 462 smokers with 24 incident cancers (RR 3.2, 90% CI 0.9-10.8, $p=0.060$) than in 1107 nonsmokers with 36 incident cancers (RR1.5, 90% CI 0.4-4.8, $p=0.300$).

The risk of prostate cancer was 4.9-fold (90% CI 1.4-17.4, $p=0.021$) among 102Val carriers compared with the wild homozygotes (Table 1). The model included maximal oxygen uptake, place of residence, serum HDL_2 cholesterol, histories of stroke and any atherosclerosis-related disease, cholesterol lowering medication, dietary iron intake and diastolic blood pressure as covariates.

The risk of cataract was examined in non-smokers, because smoking is an overwhelmingly powerful risk factor for cataracts. Among the 1107 non-smokers, the 102Val carriers had a 3.8-fold (90% CI 1.1-13.0, $p=0.038$) risk of cataract in a Cox model adjusting for blood glucose, blood leukocyte count, hair mercury content and the examination year 1989 (Table 1).

Smoking men who were PON1 102Val carriers had a 4.9-fold (90% CI 1.3-18.1, $p=0.023$) risk of cardiovascular death, compared with the 102Ile homozygotes (Table 1). The covariates included in the model were maximal oxygen uptake, history of any atherosclerosis-related disease, place of residence, serum apolipoprotein B level, plasma lipid-standardized vitamin E concentration (protective), examination year

1988 (vs. any other), and the serum fatty acid ratio (saturated/sum of monoenes and polyenes).

Among non-obese men, the PON1 102Val carriers had a 2.9-fold (90% CI 1.3-6.5, p=0.019) risk of hypertension, compared with non-carriers (Table 2), when adjusting for serum triglycerides, CHD in exercise test, dietary vitamin E intake (protective), frequency of hangovers, dietary retinol intake, and *PON1* 54 polymorphism.

As arthrosis is a chronic, gradually developing disease, only men examined in the first three years (1984-6) were included in a logistic regression analysis (Table 2). The carriers of the 102Val mutation had a 4.0-fold (90%CI 1.3-12.4, p=0.022) risk of developing an arthrosis during the follow-up, when adjusting for waist-to-hip circumference ratio, serum ferritin and dietary intakes of vitamin E and vitamin C.

Men with an 102Val allele had a 3.2-fold (90% CI 1.1-9.3, p=0.039) risk of type 2 diabetes, as compared with 102Ile homozygotes. Covariates in the model were serum fatty acid ratio (defined above), serum ferritin concentration and family history of obesity.

The Mini Mental State Examination was used to assess the presence of cognitive impairment and the degree of dementia of the KIHD participants aged 65-71 during 1998-2000. The test examines orientation (ten items), registration (three items), attention and calculation (five items), recall (three items) and language (nine items). A correct response to each item scores 1 (incorrect 0), which are summed to give a potential maximum score of 30. Higher scores indicate better cognitive function. The mean score was 25.5 (SD 2.5) among the 26 carriers of the PON102 Val allele and 26.4 (SD 2.2) among 338 non-carriers for whom data were available (one-sided p=0.031 in t-test, exact p=0.045). The Mini Mental State examination score was directly associated (Pearson's correlation coefficient 0.14, p=0.008, n=359) with serum paraoxonase enzyme activity. This association remained statistically significant (p=0.012) after a statistical adjustment for age and socio-economic status, which were other strongest predictors of the score.

Table 1: **The association of *PON1* 102Val carrier status with the risk of any cancer, prostate cancer and cardiovascular death in multivariate Cox regression models in healthy men**

Disease	Number of men free of disease at entry		Relative risk (90% CI)*	p-value
	At the start of follow-up	Who developed disease		
Any cancer**	1569	60	2.35 (1.00, 5.54)	0.052
Prostate cancer**	1569	15	4.86 (1.36, 17.36)	0.021
Cataract**	1107 non-smokers	23	3.79 (1.10, 12.98)	0.038
Cardiovascular death***	342 smokers	21	4.93 (1.34, 18.10)	0.023

* The step-up models included other strongest risk factors.
** Men with a history of cancer were excluded.
*** Men with a history of coronary heart disease or cerebrovascular stroke were excluded.

Table 2: **The association of *PON1* 102Val carrier status with the risk of hypertension and type 2 diabetes in multivariate logistic regression models in healthy men**

Disease	Number of men free of disease at entry		Relative risk (90% CI)*	p-value
	At the start of follow-up	Who developed disease		
Hypertension**	488 non-obese men	109	2.85 (1.25, 6.51)	0.019
Arthrosis***	515 men examined in 1984-6	36	3.99 (1.29, 12.36)	0.022
Type 2 diabetes****	967 non-diabetic men	33	3.17 (1.08, 9.28)	0.039

*The step-up models included other strongest risk factors.
** Men with a history of cancer or prevalent hypertension were excluded.
*** Men with a history of cancer were excluded.
**** Men with a history of cancer or prevalent diabetes were excluded.

References

1 Mackness MI, Thompson HM, Hardy AR, Walker CH. Distinction between 'A'-esterases and arylesterases. Implications for esterase classification. *Biochem J* 1987; **245:** 293-6

2 Mackness MI, Arrol S, Durrington PN. Paraoxonase prevents accumulation of lipoperoxides in low-density lipoprotein. *FEBS Lett* 1991; **286:** 152-4.

3 La Du BN, Adkins S, Kuo CL, Lipsig D. Studies on human serum paraoxonase/arylesterase. *Chem Biol Interact* 1993 Jun; 87 (1-3):25-34

4 Humbert R, Adler DA, Disteche CM, Hassett C, Omiecinski CJ, Furlong CE. The molecular basis of the human serum paraoxonase activity polymorphism. *Nature Genet* 1993; **3:** 73-6.

5 Davies HG, Richter RJ, Keifer M, Broomfield CA, Sowalla J, Furlong CE. The effect of the human serum paraoxonase polymorphism is reversed with diazoxon, soman and sarin. *Nature Genet* 1996; **14:** 334.

6 Mackness MI, Mackness B, Durrington PN, Connelly PW, Hegele RA. Paraoxonase: biochemistry, genetics and relationship to plasma lipoproteins. *Curr Opin Lipidol* 1996; **7:** 69-76.

7 Mackness MI, Arrol S, Mackness B, Durrington PN. Alloenzymes of paraoxonase and effectiveness of high-density lipoproteins in protecting low-density lipoprotein against lipid peroxidation. *Lancet* 1997; **349:** 851-2.

8 Mackness B, Durrington PN, Mackness MI. Polymorphisms of paraoxonase genes and low-density lipoprotein peroxidation. *Lancet* 1999; **353:** 468-9.

9 Shih DM, Gu L, Xia Y-R, et al. Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. *Nature 1998;* **394:** 284-7.

10 Garin MC, James RW, Dussoix P, et al. Paraoxonase polymorphism Met-Leu54 is associated with modified serum concentrations of the enzyme. A possible link between the paraoxonase gene and increased risk of cardiovascular disease in diabetes. *J Clin Invest* 1997; **99:** 62-6.

11 Mackness B, Mackness MI, Arrol S, Turkie W, Durrington PN. Effect of the molecular polymorphisms of human paraoxonase (PON1) on the rate of hydrolysis of paraoxon. *Br J Pharmacol* 1997; **122:** 265-8.

12 Mackness MI, Harty D, Bhatnagar D, Winocour PH, Arrol S, Ishola M, Durrington PN. Serum paraoxonase activity in hypercholesterolemia and insulin-dependent diabetes mellitus. *Atherosclerosis* 1991; **86**: 193-9.

13 Salonen JT. Is there a continuing need for longitudinal epidemiologic research? - The Kuopio Ischaemic Heart Disease Risk Factor Study. *Ann Clin Res* 1988; **20:** 46-50.

14 Salonen JT, Nyyssönen K, Korpela H, Tuomilehto J, Seppänen R, Salonen R. High stored iron levels are associated with excess risk of myocardial infarction in Eastern Finnish men. *Circulation 1992;* **86:** 803-11.

15 Lakka TA, Venäläinen JM, Rauramaa R, Salonen R, Tuomilehto J, Salonen JT. Relation of leisure-time physical activity and cardiorespiratory fitness to the risk of acute myocardial infarction. *N Engl J Med* 1994; **330:** 1549-54.

16 Salonen JT, Nyyssönen K, Tuomainen T-P, Mäenpää PH, Korpela H, Kaplan GA, Lynch J, Helmrich SP, Salonen R. Increased risk of non-insulin dependent diabetes mellitus at low plasma vitamin E concentrations: a four year follow-up study in men. *Brit Med J* 1995; **311:** 1124-7.

17 Ihanainen M, Salonen R, Seppänen R, Salonen JT. Nutrition data collection in the Kuopio Ischaemic Heart Disease Risk Factor Study: Nutrient intake of middle-aged eastern Finnish men. *Nutr Res* 1989; **9**: 89-95.

18 Teppo L, Pukkala E, Lehtonen M. Data quality and quality control of a population-based cancer registry. Experience in Finland. *Acta Oncologica* 1994; **33:** 365-9.

19 Everson SA, Goldberg DE, Kaplan GA, et al. Hopelessness and risk of mortality and incidence of myocardial infarction and cancer. *Psychosom Med* 1996; **58:** 113-21.

20 Salonen JT, Lakka TA, Lakka H-M, Valkonen V-P, Everson SA, Kaplan GA. Hyperinsulinemia is associated with the Incidence of hypertension and dyslipidemia in middle-aged men. Diabetes 1998; **47:** 270-275.